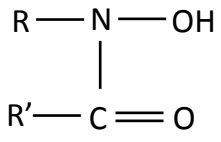
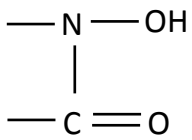
ANTIOXIDANT BEHAVIOUR AND CONFORMATIONAL ASPECTS OF DNA BINDING OF N-ARYLHYDROXAMIC ACIDS.

INTRODUCTION

Hydroxamic acids, the neutral polyfunctional molecules were first reported by H. Lossen (**1**) in 1869. In general, hydroxamic acids are the N-Aryl derivatives of hydroxylamine as illustrated in Fig.I, where R and R' are phenyl or differently substituted phenyl groups. NOH.C=O (II) is the pharmacophoric part of the molecule responsible for biological activity. These reagents show a wide spectrum of activities in analytical (**2-3**), agriculture (**4-5**), technical (**6-7**) and medicinal field (**8-9**).



I



II

Deoxyribonucleic acid is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms. DNA is a polymer. The monomer units of DNA are nucleotides, and the polymer is known as a "polynucleotide." Small molecules that bind genomic DNA have proven that they can be effective anticancer, antibiotic and antiviral therapeutic agents that affect the well-being of millions of people worldwide. The demonstration of the existence of non-covalent bonding interactions between different biomolecules (DNA, enzymes, proteins) and drugs or potentially mutagenic agents requires sensitive analytical techniques. The mode of binding of drugs to DNA can be determined by various analytical tools (**10**).

The studies on MCF-7 and A431 cells and various QSAR parameters calculated for N-Arylhydroxamic acids (**11**), show that these molecules exhibit anticancer and antitumor activity. The behaviour of binding of molecule with DNA is of great importance to understand the biological role of a molecule. In the present investigation, the mode of binding of hydroxamic acids to DNA and their conformational aspects will be determined by using following methods :

(i) UV-Vis absorption spectroscopy

(ii) Melting temperature

(iii) Fluorescence spectroscopy

(iv) Viscosity measurements

(v) Gel electrophoresis

Oxidation reactions going on within the cells can produce free radicals. In turn, these radicals can start chain reactions which can cause damage or death to the cell. Most cancer cells exhibit overproduction of ROS, which is thought to provide favourable conditions for cancer cell growth, genetic instability, and survival (**12**). Reactive oxygen species play an important role in many diseases such as cancer, atherosclerosis (**13**), etc. The intake of antioxidants has been effective in the prevention of these diseases. A variety of hydroxamic acid derivatives have been reported to have pharmacological and biological activities towards cancer, cardiovascular diseases, Alzheimer's disease, tuberculosis, etc (**14**). Since antioxidant activity of substances may have an important role in their anti-inflammatory and antiproliferative activities, the antioxidant and radical scavenging activity of the derivatives will also be evaluated in this work.

A BRIEF REVIEW OF THE WORK ALREADY DONE IN THE FIELD

The physico-chemical parameters which are known as QSAR parameters of some N-Arylhydroxamic acids are first time measured and reported from this laboratory only. The parameters determined are hydrophobic, steric and surface parameters (**15-19**) which are of further use in QSAR analysis and drug designing.

OBJECTIVES

N-Arylhydroxamic acids exhibit medicinal property as they contain a pharmacophoric structural part and Hydrogen Bond Donor (HBD) and Hydrogen Bond Acceptor (HBA) capability to bind with receptors. Work reported on some N-Arylhydroxamic acids from this laboratory show that these are effective anti-tumor agents with anti-cancer activity (**19**). It has also been proved that the bioactive molecule attack directly to DNA and kill the tumor/cancer cells (**20**). The present investigation is designed to know that

(I) WHERE AND AT WHICH POINT OF DNA THE BIOMOLECULE ATTACKS ?

(II) HOW THE INTERACTIONS BETWEEN DNA AND BIOMOLECULE TAKES PLACE ?

(III) WHAT TYPE OF CONFORMATIONAL CHANGES IN THE HIGHER ORDER STRUCTURE OF DNA TAKES PLACE ?

NOTEWORTHY CONTRIBUTION IN THE FIELD OF PROPOSED WORK

The work is already under progress in this laboratory to understand that whether the biomolecule hit the DNA ? and the answer is yes (**20**). Therefore, the present study will deal with the measurement of binding interactions and denaturation of DNA.

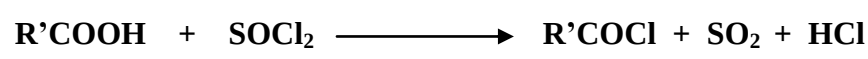
PROPOSED METHODOLOGY

The research methodology to be followed will be as under :

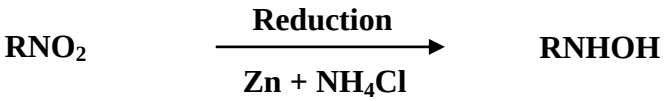
1. SYNTHESIS OF HYDROXAMIC ACIDS

N-Arylhydroxamic acids and their derivatives will be synthesized in the laboratory following the steps reported in literature (**21-22**).

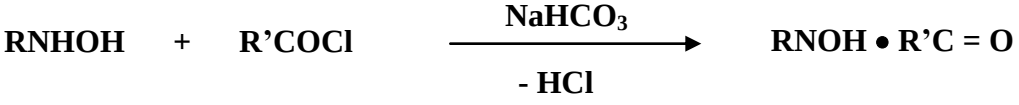
(a) Preparation of Acid Chloride



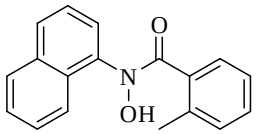
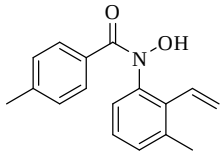
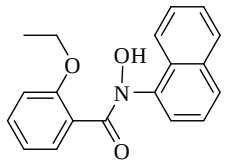
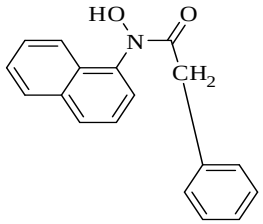
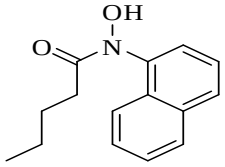
(b) Preparation of Hydroxylamines



(c) Preparation of N-Arylhydroxamic Acids



The following naphthyl derivatives of hydroxamic acids will be synthesized in the present investigation,

S. NO.	HYDROXAMIC ACIDS	MOLECULAR STRUCTURE
1.	N-naphthyl-2-methylbenzo-	
2.	N-naphthyl-4-methylbenzo-	
3.	N-naphthyl-o-n-ethoxybenzo-	
4.	N-naphthyl-phenylaceto-	
5.	N-naphthyl-valero-	

2. DNA BINDING INTERACTIONS

(i) BY UV-VIS ABSORPTION SPECTROSCOPY

The drug-DNA interaction can be detected by UV-Vis absorption spectroscopy (**23**) by measuring the changes in the absorption properties of the drug or the DNA molecules. The UV-Vis absorption spectrum of DNA exhibits a broad band (200-350 nm) in the UV region with

a maximum placed at 260 nm. This maximum is a consequence of the chromophoric groups in purine and pyrimidine moieties responsible for the electronic transitions. The molar concentration of DNA can be estimated on the basis of the measurement of the absorbance value at 260 nm. In practice, the molar concentration of DNA is evaluated in terms of the concentration of pairs of bases. The absorbance ratios (A_{260}/A_{280} and A_{260}/A_{230}) can also characterize the DNA molecules.

The **intrinsic binding constant,** K_b is a tool to investigate the magnitude of the binding strength of a compound with DNA, which represents the binding constant per DNA base pair. It can be obtained by monitoring the changes in the absorbance at the corresponding λ_{max} with increasing concentrations of DNA and is given by the ratio of the slope to the y intercept in plots [DNA] / (ε_a - ε_f) vs. [DNA], according to the following equation (**24-26**),

$$\frac{[\mathbf{DNA}]}{(\boldsymbol{\varepsilon_a} - \boldsymbol{\varepsilon_f})} = \frac{[\mathbf{DNA}]}{(\boldsymbol{\varepsilon_b} - \boldsymbol{\varepsilon_f})} + \frac{\mathbf{1}}{\mathbf{K_b}(\boldsymbol{\varepsilon_b} - \boldsymbol{\varepsilon_f})} \qquad [\mathbf{1}]$$

where,

[DNA] = Concentration of DNA in base pairs,

ε_a = Apparent absorption coefficient,

ε_b = Extinction coefficient for the free complex,

ε_f = Extinction coefficient for the complex in the fully bound form.

Slight changes in the absorption maximum as well as the molar absorptivity can be appreciated with the variations in pH or ionic strength of the media.

(ii) BY MELTING TEMPERATURE

Melting temperature (*Tm*) is the temperature value corresponding to the conversion of 50 % of the double strands into single strands, according to the equilibrium shown in **Equation (1)**.

The hypochromic effect can also be employed to verify the existence of drug-DNA interactions (**27**), due to the fact that the monitoring of the absorbance values allows studying the melting behaviour of DNA.

ds DNA $\rightleftharpoons$ **ss DNA**

For native ds-DNA, the separation of the strands starts near to T_1 and ends close to T_2. These temperature values change depending on the origin and nature of DNA (viral, bacterial, duplex, quadruplex, etc.). The temperature value corresponding to one half of DNA existing as ds-DNA and the other half as ss-DNA is named melting temperature. This value corresponds to the inflexion point in the absorbance-temperature plot (**28-29**). An increase in the absorbance value with the increase of temperature is observed because the ε (260 nm) of ss-DNA is higher than the ε (260 nm) of ds-DNA. When a drug–DNA interaction exists, Tm is shifted to values different from native ds-DNA. The magnitude of the shift depends on the type of interaction. The changes in the Tm value can be followed by techniques such as fluorescence, but UV-Vis absorption spectrometry is the most frequently employed method due to its good sensitivity, reproducibility, simplicity and versatility.

(iii) BY FLUORESCENCE SPECTROSCOPY

Fluorescence is the molecular absorption of light energy at one wavelength and its nearly instantaneous re-emission at another wavelength (usually larger). The term quenching refers to many factors that reduce or quench fluorescence. Fluorescence quenching experiments provide information concerning the localization of the drugs and their mode of interaction with DNA. The effective interaction with DNA usually causes a significant enhancement of the fluorescence intensity as well as spectral shifts (10-20 nm) in the excitation and emission spectra of drugs a consequence of different factors. The florescence titration spectra are effective for characterizing

the binding mode of the drug to DNA. The quenching of the complex by DNA follows the linear Stern-Volmer Eq (**30**),

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \qquad [2]$$

where,

F_0 = Fluorescence intensity of complex alone,

F = Fluorescence intensity of complex in the presence of DNA,

[Q] = Concentratio of the quenching reagent,

K_{SV} = Linear Stern-Volmer constant.

The K_{SV} value is obtained from the slope of the F0/F vs. [DNA] linear plot. The efficiency of quenching of fluorophore by quenching species follows Stern-Volmer relationship,

$$\log \frac{F_0 - F}{F} = \log K_b + n \log[Q] \qquad [3]$$

where,

K_b = Intrinsic binding constant,

n = Number of binding sites.

(iv) BY VISCOSITY MEASUREMENTS

Viscosity describes a fluid's internal resistance to flow and may be thought of as a measure of fluid friction. This resistance becomes apparent when a layer of fluid is made to move in relation to another layer. Viscosity is a principal parameter when any flow measurements of fluids, such as liquids, semi-solids, gases and even solids are made. The greater the friction, the greater the amount of force required to cause this movement, which is called shear. The velocity gradient, dv/dx, is a measure of the change in speed at which the intermediate

layers move with respect to each other. It describes the shearing the liquid experiences and is thus called shear rate. The viscosity of DNA solution increases due to the binding of drug molecules. Viscosity experiment is an effective tool to determine the binding mode of small molecules and DNA. The relative specific viscosity will be obtained by Eq. (**31-32**),

$$\eta_r = \sqrt[3]{\frac{\eta}{\eta_0}} \qquad [4]$$

where,

η_0 = Specific viscosity contributions of DNA in the absence of the complex,

η = Specific viscosity contributions of DNA in the presence of the complex.

3. CONFORMATIONAL ASPECTS OF DNA INTERACTIONS

(i) BY GEL ELECTROPHORESIS

Electrophoresis is the movement of a charged particle in an electrical field which enables the sorting of molecules based on size and charge. Gel electrophoresis refers to using a gel as an anticonvective medium during electrophoresis. The DNA molecules can be made to move through the agarose gel in the presence of suitable dyes. The results will be analysed by visualizing the gel with UV transilluminator (**33-34**).

(ii) FLOURESCENCE MICROSCOPY

Flourescence microscope is basically a light microscope that uses the fluorescence light eminating from fluorescent dyes within the sample for imaging. Flourescence microscopy is a unique way of using a microscope to discover facts about specimen that often are not known by standard bright field microscopy. Here, specimen are self-illuminated by internal light, so bright

objects are seen more easily. It can be used to find out the effect of the molecules on the higher-order structure of DNA through single-molecule observed in solution.

The diffusion constant, D for each DNA molecule will be calculated by using the following eq. (**35-37**),

$$(\mathbf{R(t) - R(0)^2}) = \mathbf{4Dt + At^2} \qquad [\mathbf{5}]$$

where,

R(t)	=	(R_x, R_y) is the position of the centre of mass for a DNA,
$(R(t) - R(0)^2)$	=	Mean square displacement,
A	=	Numerical constant related to convective flow,

The effective hydrodynamic radius of a single DNA molecule will be obtained from the Stokes-Einstein relationship given in eq. (**38-39**),

$$\mathbf{R_H = \frac{k_B T}{6\pi\eta_s D}} \qquad [\mathbf{6}]$$

where,

k_B	=	Boltzmann constant and
η_s	=	Viscosity of the solvent.

4. ANTIOXIDANT ACTIVITY DETERMINATION

A number of assays have been introduced for the measurement of the total antioxidant activity of pure compounds (**40**). Each method relates to the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time point or over a range. Two types of approach have been taken, namely, the inhibition assays in that the extent of the scavenging by hydrogen-or

electron-donation of a pre-formed free radical is the marker of antioxidant activity, as well as assays involving the presence of antioxidant system during the generation of the radical. The following methods will be applied to measure the antioxidant property of PBHA (**41**-**42**).

1) Free Radical Scavenging Method
2) β-Carotene-Linoleate Method
3) DNA Cleavage Method

EXPECTED OUTCOME OF THE PROPOSED WORK

Generally, organic molecules are less soluble in water but are more lipophilic and more permeable through membrane in their unionized form. It has been reported that biological activity of hydroxamic acids may be due to the similar structure present in proteins (**43**). Since, hydroxamic acids contain the smallest unit –C=O.NH, that can bind to DNA helix, therefore knowledge of favoured binding site and conformational aspects as well as its antioxidant properties are also important to understand the role played by hydroxamic acids in biological processes and cancer drug designing.

This piece of research work will be a small step for fulfilling the quest of knowledge regarding the DNA binding capacity and various related parameters of naphthyl derivatives of hydroxamic acids. It will be a continuation of the work done and reported from this laboratory on "QSAR and Drug Dsigning for Anti-tomor / Anti-cancer Activity".

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